Antisense Oligonucleotides Against Thrombospondin-1 Inhibit Activation of TGF- β in Fibrotic Renal Disease in the Rat *in Vivo*

Christoph Daniel,* Yoshitugu Takabatake,[†]
Masayuki Mizui,[†] Yoshitaka Isaka,[†]
Hiroshi Kawashi,[‡] Harald Rupperecht,[§] Enyu Imai,[†]
and Christian Hugo*

From the Division of Nephrology,* Universität Erlangen-Nürnberg, Erlangen, Germany; Medizinische Poliklinik,[†] LMU University, Munich, Germany; the Institute of Nephrology,[‡] Niigata University School of Medicine, Asahimachi-dori, Niigata, Japan; and the 1st Department of Medicine,[§] University School of Medicine, Osaka, Japan

Specific treatment of chronic progressive renal disease is very limited. TGF- β , considered as the major cytokine causing tissue scarring, must be activated extracellularly before it can bind to its receptors. Thrombospondin-1 (TSP1) has been identified as an activator of latent TGF- β in *in vitro* systems and in pancreas and lung homeostasis in mouse pups *in vivo*, but whether this is also true in inflammatory fibrotic disease is unknown. We examined a rat model of mesangial proliferative glomerulonephritis, where TGF- β has been demonstrated to mediate renal fibrosis. In this study, antisense phosphorothioate oligonucleotides against TSP1 were successfully transferred into almost all glomeruli of perfused diseased kidneys and markedly inhibited *de novo* synthesis of TSP1. This effect was accompanied by decreased activation but not expression of TGF- β and by the inhibition of the TGF- β -dependent smad-signaling pathway, as well as transcription of TGF- β target genes such as EDA-fibronectin, resulting in a markedly suppressed accumulation of extracellular matrix. In sharp contrast, neither glomerular cell proliferation nor influx of macrophages was affected by this therapy in experimental mesangial proliferative glomerulonephritis. These results demonstrate that TSP1 is the major endogenous activator of TGF- β in experimental inflammatory kidney disease. (*Am J Pathol* 2003, 163:1185–1192)

During renal disease progression, extracellular matrix accumulation is the common hallmark of basically any renal process causing end-stage renal failure in man. Currently no specific and effective antifibrotic therapy for chronic progressive renal disease is available. Mesangial proliferative

glomerulonephritis, the most common type of glomerulonephritis in the western world,¹ is characterized by mesangial cell (MC) proliferation, activation, extracellular matrix expansion,² and progression to end-stage renal disease for up to 50% of the patients.³

The role of TGF- β as a major profibrotic cytokine in various fibrotic diseases in multiple organ systems and in particular in experimental renal disease has been well established.⁴ TGF- β mRNA and protein are increased in experimental mesangial proliferative glomerulonephritis in the rat, the anti-Thy1 model.⁵ Direct blockade of TGF- β action by multiple techniques markedly reduced extracellular matrix accumulation,^{6–9} while mice transgenic for an active form of TGF- β 1 developed progressive renal disease characterized by MC matrix accumulation and interstitial fibrosis.¹⁰ Evidence for the central importance of TGF- β in mediating fibrosis in human kidney disease is well supported by the widespread correlation of TGF- β up-regulation with extracellular matrix excess in any type of human kidney disease.¹¹ While these studies suggest great benefit from suppression of TGF- β function in fibrotic kidney disease, it must be taken into account that TGF- β is a multifunctional cytokine that exhibits other essential functions in mammals. Mice lacking either the TGF- β 1, -2, or -3 gene do not survive beyond a few weeks after birth,^{12–14} demonstrating that accurate regulation of TGF- β seems to be critical for the health of mammals. Therefore, any anti-TGF- β 1 therapeutic approach should target the local overproduction (function) of TGF- β as specifically as possible.¹²

Considering the widespread distribution of TGF- β in most cell types, TGF- β action is best controlled via its local activation process.^{11,15–17} TGF- β is secreted by most cell types as a latent, inactive procytokine complex that consists of the mature, active TGF- β protein non-covalently bound to a dimer of its N-terminal propeptide, the so-called latency-associated protein (LAP), and vari-

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Address reprint requests to Christian Hugo, M.D., Division of Nephrology, Universität Erlangen-Nürnberg, Loschgestr. 8, 91054 Erlangen, Germany. E-mail: Christian.Hugo@rzmail.uni-erlangen.de.

Figure 1. Experimental design of the study.

ably to a latent TGF- β binding protein (LTBP).¹⁵ While various players/mechanisms have been identified to activate TGF- β under *in vitro* conditions, very little is known about TGF- β activation in an inflammatory process *in vivo*.^{15–17}

Recent data suggest that the homotrimeric extracellular-matrix protein TSP1 is an activator of TGF- β 1 *in vitro* in different cell systems, including MC, as well as in cell-free systems.^{18–20} It has been demonstrated that TSP1 forms a trimolecular complex with the latent TGF- β procytokine leading to a non-proteolytic activation process that allows binding of TGF- β to its receptors.^{17–20} Comparing TSP1 null mice with TGF- β 1 null mice, TSP1 was identified as a major activator of TGF- β 1 in pancreas and lung homeostasis in mice pups *in vivo*.^{21,22} Nevertheless, the phenotype of TSP1 null mice is much milder compared to TGF- β 1 null mice, TSP1 null mice are healthy and have a normal life expectancy.^{21,22} TSP1 expression *in vitro* is regulated by various cytokines such as PDGF, FGF-2, or TGF- β , and is frequently expressed *de novo* at sites of inflammation and wound healing.²³ In various experimental renal disease models, TSP1 expression co-localizes with TGF- β and predicts the development of tissue fibrosis.²⁴ In the anti-Thy1 model of mesangial proliferative glomerulonephritis, a marked transient *de novo* expression of TSP1 by MC is regulated by FGF-2 and PDGF and coincides with the up-regulation of TGF- β 1.^{5,25}

In this study, we examined the role of TSP1 in experimental inflammatory renal disease as a potential endogenous activator of TGF- β . Therefore, by using the hemagglutinating virus of Japan (HVJ)-liposome and the electroporation method of transferring phosphorothioate oligonucleotides (ODN) against TSP1 into renal glomeruli *in vivo*, we investigated whether specific inhibition of TSP1 would be a feasible approach for the treatment of inflammatory renal disease *in vivo*.

Materials and Methods

Phosphorothioate Oligonucleotides against TSP1 in MC in Vitro

Antisense and control (scrambled) phosphorothioate oligonucleotides of 14 to 25 base-length against TSP1 were designed using A and T rich sequences avoiding accumulation of more than 4 G/C bases and alignments to other known sequences. Oligonucleotides were manufactured by MWG Biotech (Ebersberg, Germany).

Figure 2. Transfer of phosphorothioate ODN against TSP1 in cultured MC inhibits glomerular TSP1 expression. FITC-labeled phosphorothioate ODNs against TSP1 were successfully transferred into nuclei of cultured MC (A). Of 11 non-cross-reacting antisense sequences, five ODNs were able to block FCS-mediated TSP1 induction in cultured MC compared to stimulated cells using transfection reagent without ODNs (pC) (B). Non-stimulated but Effectene-treated MC expressed no TSP1 (nC). The two most effective ODN sequences (number 2 and 11) were chosen for the *in vivo* experiments.

Eleven different non-cross-reacting antisense sequences against TSP1 were designed and tested regarding its ability to block fetal calf serum (FCS)-mediated TSP1 induction in cultured MC (Figure 2). After starvation for 48 hours in serum-free Dulbecco's modified Eagle's medium (DMEM), MC were transfected with 8 μ mol/L ODNs using Effectene transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's instructions. Transfection efficacy was monitored using fluorescein isothiocyanate (FITC)-labeled ODNs. TSP1 expression was induced by addition of 10% FCS 2 hours after transfection. Controls were treated with Effectene alone with (pC) or without (nC) addition of FCS. Toxic effects after transfection with ODNs could not be observed.

Animal Experiment

The experimental design is shown in Figure 1. Experimental mesangial proliferative glomerulonephritis was induced in 6 to 8 Sprague-Dawley rats per group (150 to 200 g; Charles River, Sulzfeld, Germany) by a single injection of 1 mg/kg of the mouse monoclonal anti-Thy1 antibody 1–22–3. On day 2 after disease induction, *de novo* expression of TSP1 was targeted by the selective transfer of specific phosphorothioate ODN into renal glomeruli via left renal artery perfusion. Either the HVJ-liposome method or electroporation of the left kidney using oval-shaped electrodes and electric pulses (six 75-V pulses of 100-ms duration each with 900-ms intervals) was used as described recently.²⁶ Pilot studies using Cy3-labeled antisense oligonucleotides against TSP1 investigated the effectiveness of the ODN transfer in the left kidney via electroporation or via the HVJ-liposome method at a 10' biopsy. At the same time, the non-perfused right kidneys were also examined regarding potential transfer of Cy3-positive ODNs via the circulation. On day 7, parts of the perfused left kidneys were also used for glomerular isolation,²⁵ total RNA prepara-

tion, and real-time RT-PCR evaluation for EDA-fibronectin mRNA.

Since only a single kidney (left) was selectively affected by the transfer of TSP1 antisense oligonucleotides, functional parameters such as proteinuria and creatinine clearance were not determined. In renal biopsies, the number of infiltrating macrophages, the number of proliferating MC, the expression of the contractile protein α -smooth-muscle actin as a marker of MC activation, and the expression of active TGF- β , total TGF- β 1, total TGF- β 2, TSP1, and the glomerular number of nuclei positive for the phosphorylated Smad2/3 protein as a marker of TGF- β activation, as well as of extracellular-matrix proteins such as collagen I, collagen IV, and fibronectin were determined.

A second independent experiment was performed in an identical manner as described above using six rats per group. Seven days after disease induction, glomeruli of the left (antisense- or scrambled-treated) or right (control) kidney were isolated and glomerular TGF- β activity determined using an established PAI-1 luciferase bioassay system,²⁷ as described below.

Renal Morphology and Immunohistochemistry

Renal biopsies were fixed in methyl Carnoy's solution, embedded in paraffin, and cut into 5- μ m sections for indirect immunoperoxidase staining as described elsewhere.^{24,25}

The following antibodies were used in this study: a murine IgM monoclonal antibody (mAb) against the proliferating cell nuclear antigen (PCNA) (19A2; Coulter Immunology, Hialeah, FL); ED-1, a murine IgG₁ mAb to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (Serotec Ltd., Oxford, UK); OX-7, a murine IgG₁ mAb specific for mesangial cells (Serotec); and α -smooth-muscle actin, a murine IgG₂ mAb specific for activated MC (Sigma Chemical Co, St. Louis, MO). Immunostaining for matrix proteins was conducted with polyclonal antibodies to collagen I (rabbit anti-rat collagen I; Quartett, Berlin, Germany), collagen IV (goat anti-human/bovine collagen IV; Southern Biotechnology Associates, Inc., Birmingham, AL), active TGF- β 1 (chicken anti-human active TGF- β 1; R&D systems, Wiesbaden-Nordenstadt, Germany),²⁸ TGF- β 1 (rabbit anti-human TGF- β 1; Santa Cruz Biotechnology Inc., Santa Cruz, CA),²⁴ TGF- β 2 (rabbit anti-human TGF- β 2; Santa Cruz Biotechnology),²⁴ and a murine IgG₁ mAb against TSP1 (Dunn, Labortechnik GmbH, Asbach, Germany), P-Smad2/3 (rabbit anti-human Smad2 peptide phosphorylated at Ser-433/435; Santa Cruz). Negative controls for immunostaining included either deleting the primary antibody or substituting the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or preimmune rabbit/goat IgG.

For each biopsy, 40 to 70 cortical glomerular cross-sections containing more than 20 discrete capillary segments each were evaluated in a blinded fashion as described previously.^{25,31} Two different scores were used for quantification. The first one ranged from 0 to 4; 0 =

glomerulus without any positive staining, 1 = glomerulus with up to 25% positive staining, 2 = glomerulus with 26% to 50% positive staining, 3 = glomerulus with 51% to 75% positive staining, 4 = glomerulus with 76% to 100% positive staining. For the 0 to 10 score the biopsies were scored in 10% ranges. In addition, most parameters have also been evaluated by computerized morphometry (Visi-tron Systems GmbH, Puchheim, Germany). In addition, the average number of ED-1-positive macrophages per glomerular cross-section was determined.

Immunohistochemical Double-Staining

To determine the number of proliferating MC, double immunostaining for PCNA, a marker of cell proliferation, and for OX-7 (MC-specific) was performed as described previously.²⁵

TGF- β Activity

Since detergents used for protein extraction of glomeruli may lead to unspecific activation of TGF- β , isolated glomeruli (8000/ml medium) from day 7 animals were incubated for a 24-hour period in assay medium (DMEM supplemented with L-glutamine and penicillin/streptomycin) at 37°C and 5% CO₂. The amount of secreted active (direct) and total (after heat stimulation at 80°C for 10 minutes) TGF- β in glomerular supernatants was determined by an established bioassay using PAI-1 luciferase reporter cells as described previously.²⁷ Mink lung epithelial cells (clone 32) stably transfected with the TGF- β response element of the human plasminogen activator inhibitor-1 (PAI-1) gene promoter fused to firefly luciferase reporter gene were a generous gift from Dr. D. B. Rifkin (New York University Medical Center). Cells were maintained in DMEM supplemented with 10% calf serum, L-glutamine, and 200 μ g/ml G418. Briefly, Mink lung epithelial cells were plated into 96-well tissue culture plate at 2.2×10^5 cells/ml and incubated for 3 to 4 hours for optimal attachment. After aspiration of the growth medium from the attached cells, 50 μ l of assay medium and 50 μ l of sample or standard were added followed by overnight incubation at 37°C. After incubation, cells were lysed with lysis buffer at room temperature for 20 minutes. Lysates were analyzed for luciferase activity using a luminometer after the injection of 100 μ l of substrate solution and recorded as relative light units. The mean values of triplicate samples were converted into concentrations of TGF- β using a standard curve (0.5 to 500 pmol) obtained with human recombinant TGF- β 1 (R&D Systems).

Real-Time Quantitative RT-PCR

RNA, used for quantitative RT-PCR, was purified from isolated glomeruli pooled from either antisense-ODN- or scrambled-ODN-treated kidneys using Trizol following manufacturer's instructions and treated with DNase to avoid DNA contamination. Reverse transcription was performed using TaqMan reverse-transcription reagents

(Applied Biosystems, Weiterstadt, Germany) following manufacturer's instructions.

Real-time RT-PCR was performed on a TaqMan ABI 7000 Sequence detection system using the Mastermix (all from Applied Biosystems). After an initial hold of 3 minutes 95°C samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. The cDNA content of each sample was compared with β -actin as a housekeeping gene following the $\Delta\Delta C_t$ technique.²⁹ For EDA-fibronectin a fluorescence-labeled probe was used (forward primer 5'-TCAGAACCGGAACGGAGAAA-3'; reverse primer 5'-ACATACGTGAATGCCAGTCCTTT-3'; FAM/TAMRA-labeled probe 5'-TGGTTCAGACTGCAGTGACCAACATTGA-3'). Housekeeping gene β -actin was quantified using the SYBR-Green method (forward primer 5'-CTGGTGTGGATTGGTGGCTCTA; reverse primer 5'-CTGCTTGCTGATCCACATCTG-3').

Statistical Analysis

All values are expressed as mean $\pm$ SD. Statistical significance (defined as $P < 0.05$) was evaluated using Student's *t*-test or one-way analysis of variance with modified *t*-test using the Bonferroni method.

Results

Transfer of Phosphorothioate ODN against TSP1 in Cultured MC Inhibits Glomerular TSP1 Expression

Using effectene as a transfer reagent, FITC-labeled phosphorothioate ODNs against TSP1 were successfully transferred into nuclei of cultured MC (Figure 2A). Transfection efficacy was about 80% of all cultured MC. Of eleven non-cross-reacting antisense sequences, five ODNs were able to block 10% of FCS-mediated TSP1 induction in cultured MC (Figure 2B, numbers 2, 4, 8, 10, 11).

Transfer of Phosphorothioate ODN against TSP1 into Glomerulonephritic Rats Inhibits Glomerular TSP1 Expression

The two most effective ODN sequences (5'-TTCTCCGTGTGATTGAA-3', 5'-CACCTCCAATGAGTT-3') in cultured MC were chosen for the *in vivo* experiments and compared to scrambled control oligos (5'-TGTTATCGAGTTCGATT-3, 5'-ACATTCGCTTCACGA-3') (Figure 3). In pilot studies, successful transfer of Cy3-labeled phosphorothioate ODN against TSP1 was demonstrated in almost 100% of glomeruli (Figure 3A) on day 2 of nephritis by the renal electroporation method, while the HVJ-liposome method only targeted about 70% of the glomeruli.²⁸ Therefore, all data presented regarding transfer of oligonucleotides against TSP1 stem from experiments applying the electroporation method on day 2 and collecting both the perfused left kidney and the non-perfused right kidney on day 7. Since renal artery

Figure 3. Transfer of phosphorothioate ODN against TSP1, but not scrambled ODN, into glomerulonephritic rats inhibits glomerular TSP1 expression. In all figures treated kidneys (solid bars) were compared to nontreated control kidneys (open bars). On day 2 after disease induction, successful transfer of Cy3-labeled phosphorothioate ODN against TSP1 was demonstrated in almost 100% of glomeruli (A) of the left kidney, but none of the right kidney (not shown). Transfer of antisense oligonucleotides selectively inhibited *de novo* expression of glomerular TSP1 protein in the perfused left kidney on day 7 by more than 60% compared to the non-perfused right kidney, while gene transfer of scrambled control oligonucleotides did not alter glomerular TSP1 expression (B, evaluated by scoring; C, evaluated by computerized morphometry). Representative examples of unaltered TSP1 expression by scrambled ODNs (D) versus reduced TSP1 expression by antisense ODNs (E) are demonstrated by the extent of dark gray immunostaining within glomeruli. The * marks significant differences ($P < 0.01$) of antisense groups versus the control (scrambled) group.

perfusion is solely done via the left kidney and no Cy3-positive fluorescence from ODNs was detected in the contralateral right kidney, the right kidney functions as an ideal internal control for this treatment in addition to the scrambled control ODNs. Transfer of antisense oligonucleotides (using two different types) selectively inhibited

glomerular TSP1 protein content (by immunostaining) of the left kidney on day 7 by more than 60%, compared to the non-perfused right kidney, while gene transfer of scrambled control oligonucleotides did not alter glomerular TSP1 expression (Figure 3, B to E).

Transfer of Phosphorothioate ODN against TSP1 Decreases Activation but Not Expression of TGF- β in Nephritic Glomeruli

If the *de novo*-expressed TSP1 activates TGF- β in the anti-Thy1 model, glomerular TGF- β activity but not total TGF- β expression should be reduced in treated rats. Glomerular TGF- β 1 or TGF- β 2 protein (by immunostaining) was not changed in any group of nephritic rats, confirming specific targeting of TSP1 by gene therapy (Figure 4, A and B). In addition, no change in the glomerular total TGF- β 1 content of the left versus right kidney was detected in either scrambled- or antisense-treated rats when the PAI-1 luciferase reporter bioassay system was used (Figure 4C). In contrast, TSP1 antisense but not scrambled therapy reduced the active fraction of glomerular TGF- β secretion by 50% compared to the untreated right kidney (Figure 4C). In addition, active TGF- β in nephritic glomeruli was determined by two different immunostaining methods: First, using an antibody recognizing the active form of TGF- β 1 (Figure 4D) and secondly, using an antibody specific for the phosphorylated form of the TGF- β signal-transduction molecule Smad 2/3 (Figure 4H). In agreement with the bioassay results, antisense but not scrambled ODN therapy was associated with a markedly decreased glomerular TGF- β activity in the left kidney as reflected by immunostaining for active TGF- β 1 (Figure 4E, using a semiquantitative scoring system; and 4F, by computerized morphometry) and by a marked reduction of glomerular cells showing positive nuclei for the TGF- β -signaling molecule phospho-Smad2/3 (Figure 4G).

Transfer of Phosphorothioate ODN against TSP1 Inhibits Glomerular Extracellular Matrix Accumulation and MC Activation

Next, the influence of TSP1-blocking therapy on a typical TGF- β -dependent matrix gene such as EDA-fibronectin was investigated using real-time PCR detection from nephritic glomeruli. Antisense therapy against TSP1 markedly inhibited transcript expression of EDA-fibronectin in isolated glomeruli from nephritic rats on day 7 (Figure 5A). In addition, glomerular ECM accumulation on day 7 as determined by immunostaining for collagen IV was inhibited by antisense but not by scrambled ODN therapy against TSP1 (Figure 5, C to F). Similar results were obtained by evaluating the glomerular accumulation of collagen I in antisense- or scrambled-treated animals (Figure 5, G and H). Glomerular *de novo* expression of smooth-muscle actin during glomerulonephritis is considered a sign of MC activation. Decreased glomerular

Figure 4. Transfer of phosphorothioate ODN against TSP1 decreases activation, but not expression of TGF- β in nephritic glomeruli. In all figures treated kidneys (solid bars) were compared to nontreated control kidneys (open bars). Glomerular TGF- β 1 or TGF- β 2 protein (by brown immunostaining) was not changed in any group of nephritic rats (A and B). In agreement, equal levels of total TGF- β levels were determined using the PAI-1 luciferase assay. Active TGF- β levels were significantly reduced in the antisense-treated kidneys (C). Additionally, active TGF- β in nephritic glomeruli was determined by an antibody specifically recognizing the active form of TGF- β 1 (D, gray cytoplasmic staining) and by an antibody specific for the phosphorylated form of the TGF- β signal-transduction molecule Smad 2/3 (H, black nuclear staining; arrowheads indicate examples for P-Smad2/3-positive nuclei). Inhibition of TSP1 expression after antisense ODN therapy but not scrambled ODN therapy was associated with a markedly decreased glomerular TGF- β activity in the left kidney as reflected by immunostaining for active TGF- β 1 (E, evaluated by scoring system; F, evaluated by computerized morphometry) and by a marked reduction of glomerular cells showing positive nuclei for the TGF- β -signaling molecule phospho-Smad2/3 (G). * marks significant differences ($P < 0.01$) of antisense groups versus the control (scrambled) group.

TGF- β activity by the TSP1 antisense- but not by scrambled-ODN transfer technique was accompanied by a marked reduction in MC activation determined by smooth-muscle-actin staining (Figure 5B).

Figure 5. Transfer of phosphorothioate ODN against TSP1 inhibits glomerular extracellular matrix accumulation and MC activation. In all figures treated kidneys (solid bars) were compared to nontreated control kidneys (open bars). EDA-fibronectin mRNA, a typical TGF- β -dependent matrix gene, was quantified via quantitative real-time PCR in pooled isolated glomeruli comparing scrambled- with antisense-treated kidneys. Antisense therapy against TSP1 almost completely abolished transcript expression of EDA-fibronectin in isolated glomeruli from nephritic rats on day 7 (A). Glomerular ECM accumulation on day 7 as determined by immunostaining for collagen IV (C and D) or collagen I (G and H) was markedly inhibited by antisense but not by scrambled ODN therapy against TSP1. E: A representative picture of glomerular collagen IV accumulation (dark gray immunostaining) in antisense-treated rats; F shows typical collagen IV expression in scrambled-treated rats on day 7. Glomerular *de novo* expression of smooth-muscle actin during glomerulonephritis, a marker of MC activation, was decreased by the TSP1 antisense but not by scrambled ODN transfer (B). Matrix accumulation was either evaluated by scoring system (C and G) or by computerized morphometry (B, D, H). * marks significant differences ($P < 0.01$) of antisense groups versus the control (scrambled) group.

Transfer of Phosphorothioate ODN against TSP1 Affects Neither Glomerular Proliferation Nor Influx of Macrophages in Nephritic Rats

Since TGF- β has been shown to inhibit MC proliferation *in vitro*³⁰ we also examined if a reduced TGF- β activity in

nephritic animals is accompanied by an increased proliferative response of MC *in vivo*. Despite alteration of TGF- β activity and matrix formation, MC proliferation (by double immunostaining for PCNA and OX-7) was unchanged by either TSP1 antisense therapy (left kidney 7.6 ± 1.1 versus untreated right kidney 7.8 ± 1.3) or scrambled therapy (left kidney 7.2 ± 0.6 versus untreated right kidney 7.2 ± 0.5).

Although TGF- β is a potential chemotactic attractant for monocytes/macrophages,³¹ the number of ED-1-positive monocytes/macrophages per glomerular cross-section were not altered by either TSP1 antisense therapy (left kidney 1.5 ± 0.3 versus untreated right kidney 1.8 ± 0.5) or scrambled therapy (left kidney 1.6 ± 0.4 versus untreated right kidney 1.9 ± 0.3).

Discussion

TGF- β is considered the major cytokine that causes tissue fibrosis in many different inflammatory disease processes, in particular in renal disease.⁴ TGF- β is secreted by most cells as a latent procytokine complex that requires extracellular activation before it can interact with its receptors.¹⁵ Despite great interest in therapeutic anti-TGF- β strategies to treat fibrotic disease, the mechanism of TGF- β activation in an inflammatory process *in vivo* is still incompletely defined.

The major finding in the current study was that TSP1 is an important endogenous activator of TGF- β in this experimental inflammatory kidney disease model. Antisense phosphorothioate ODN against TSP1 were successfully transferred into almost all glomeruli of perfused diseased kidneys and markedly inhibited *de novo* synthesis of TSP1. This effect was accompanied by decreased activation but not expression of TGF- β , and by the inhibition of the TGF- β -dependent smad-signaling pathway as well as transcription of TGF- β target genes such as EDA-fibronectin, resulting in a markedly suppressed accumulation of extracellular matrix. In sharp contrast, glomerular endothelial or mesangial cell proliferation was not affected by this therapy in experimental mesangial proliferative glomerulonephritis.

Comparing the degree of TSP1 inhibition via this therapy with the decrease of glomerular TGF- β activation and extracellular matrix formation in this experiment, the data presented here suggest that TSP1 is not just one of many, but rather is the major activator of TGF- β in this model. Nevertheless, participation of other activators of TGF- β or direct secretion of the active cytokine by glomerular cells cannot be completely excluded. The beneficial effects as demonstrated in this study are in agreement with previous studies antagonizing TGF- β by antibodies, decorin injections, or gene therapy.⁶⁻⁹

Nevertheless, therapeutic antifibrotic strategy targeting TSP1-mediated activation of TGF- β rather than non-specific, systemic blockade of TGF- β ligand-receptor interactions may have an advantageous side-effect profile, because basal TGF- β activity and/or alternate activation pathways of TGF- β in other systems/organs may not be affected. A general and complete inhibition/lack of TGF- β

can lead to serious consequences, while a general and complete inhibition of TSP-1 may not. This concept is supported by the major differences in the biology of TSP1 and TGF- β 1 null mice.^{12,21,22} Due to a multifactorial dysregulation of the immune system,¹² TGF- β 1 null mice show a generalized, excessive autoinflammatory phenotype that results in early death. The contrasting mild phenotype and normal life-span of TSP1 null mice may reflect the fact that these animals show a reduction but not a complete lack of TGF- β activity in some organs.²¹ In addition, mice with deletion of one allele of TGF- β 1 and reduced TGF- β 1 levels have an increased cell turnover and a susceptibility to tumorigenesis in liver and lung,³² which is lacking in the TSP1 null mice. Maximal specificity of the anti-TSP1 treatment relates to the fact that TSP1-mediated TGF- β activation requires a direct interaction of secreted TSP1 and TGF- β in a complex extracellular neighborhood and that TSP1 is tightly regulated in disease. In contrast, the latent TGF- β procytokine-complexes and the TGF- β receptors are highly and widely expressed in most tissues,³³ but little TGF- β is present in its biologically active form, supporting the concept of critical regulation of TGF- β action by its activators. This concept is additionally supported by the fact that *in vivo* gene transfer of the constitutively active TGF- β 1 gene into the lung of rats caused extensive fibrosis, while overexpression of the latent TGF- β 1 transgene did not.³⁴ In this context, it is interesting that even in glomeruli of anti-Thy1 diseased rats with increased matrix production, most of the TGF- β appears to be still in its latent form and that the reduction of the active fraction by about 50% via TSP1 inhibition was able to markedly reduce matrix accumulation. TSP1 perfectly fits into the role of a tightly regulated, local activator of TGF- β that is induced by other cytokines such as PDGF and FGF-2 as well as potentially TGF- β in response to glomerular injury,²⁵ while in the normal rat glomerulus, TSP1 expression is below detection level.

TSP1 is able to activate TGF- β 1 and TGF- β 2.³⁵ Since both glomerular TGF- β 1 and TGF- β 2 are increased in the anti-Thy1 model, it cannot be excluded by this study that the effect of the blocking peptide treatment is due to inactivation of both TGF- β 1 and/or TGF- β 2. Nevertheless, the therapeutic effect seen by TGF- β 1 inhibition in previous studies^{6,8} was very similar to the effects of blocking TSP1 as shown here.

MC proliferation is characteristic of many glomerular diseases and frequently linked to extracellular matrix accumulation.² Although TGF- β inhibits cell proliferation *in vitro* in different cell types including MC^{30,36} and has been suggested as a potential endogenous inhibitor of MC proliferation in glomerulonephritis, the pathophysiological role of TGF- β in regard to mesangial cell proliferation in experimental glomerulonephritis is still controversially discussed.^{4-9,11,36} Despite reducing TGF- β activity, TSP1 antisense-ODN treatment did not affect the proliferative response of the diseased glomeruli, suggesting that cellular proliferation and matrix accumulation/fibrosis can be dissociated and that TGF- β is not an endogenous inhibitor of glomerular proliferation in this glomerulonephritis model.

Influx of monocytes/macrophages into the glomerulus is also an important feature of glomerular disease. *In vivo* and *in vitro* studies have shown that TGF- β 1 can be chemotactic for mononuclear cells and it can reduce macrophage adhesiveness, which potentially leads to increased clearance from inflammatory sites.³¹ Nevertheless, TSP1 antisense treatment in this study did not affect macrophage accumulation in glomeruli in the anti-Thy1 model.

In conclusion, TSP1 is a tightly regulated major endogenous activator of TGF- β in an inflammatory glomerulonephritis model in the rat. The TSP1-mediated activation of TGF- β is responsible for the major part of the glomerular matrix formation occurring in this model, but does not affect MC/GEN proliferation or macrophage accumulation. The widespread link of TSP1 and TGF- β in several different experimental renal disease models as well as in human kidney disease^{11,24,37} suggests a central role of TSP1 in mediating renal/tissue fibrosis through interaction with latent TGF- β , but future studies have to prove this concept in a broader range of fibrotic diseases. Nevertheless, the specific inhibition of TSP1-mediated TGF- β activation in inflammatory disease as demonstrated in this study may prove to be an especially favorable therapeutic approach considering the well-known long-term side effects of a generalized non-specific blockade/deletion of TGF- β in mice.

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